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Jeffrey J. Bednarski  
*Washington University School of Medicine*

Ruchi Pandey  
*Washington University School of Medicine*

Emily Schulte  
*Washington University School of Medicine*

Lynn S. White  
*Washington University School of Medicine*

Bo-Ruei Chen  
*Washington University School of Medicine*

*See next page for additional authors*

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RAG-mediated DNA double-strand breaks activate a cell type–specific checkpoint to inhibit pre–B cell receptor signals

Jeffrey J. Bednarski,1* Ruchi Pandey,2* Emily Schulte,1 Lynn S. White,1 Bo–Ruei Chen,2 Gabriel J. Sandoval,2 Masako Kohyama,2 Malay Haldar,2 Andrew Nickless,1 Amanda Trott,1 Genhong Cheng,3 Kenneth M. Murphy,2 Craig H. Bassing,1 Jacqueline E. Payton,2 and Barry P. Sleckman2

1Department of Pediatrics and 2Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO 63110
2Department of Microbiology, Immunology and Molecular Genetics, University of California, Los Angeles, CA 90095
3Division of Cancer Pathobiology, Department of Pathology and Laboratory Medicine, Center for Childhood Cancer Research, Children’s Hospital of Philadelphia, Philadelphia, PA 19104

DNA double-strand breaks (DSBs) activate a canonical DNA damage response, including highly conserved cell cycle checkpoint pathways that prevent cells with DSBs from progressing through the cell cycle. In developing B cells, pre–B cell receptor (pre–BCR) signals initiate immunoglobulin light (Igl) chain gene assembly, leading to RAG-mediated DNA DSBs. The pre–BCR also promotes cell cycle entry, which could cause aberrant DSB repair and genome instability in pre–B cells. Here, we show that RAG DSBs inhibit pre–BCR signals through the ATM and NF–κB–dependent induction of SPIC, a hematopoietic–specific transcriptional repressor. SPIC inhibits expression of the SYK tyrosine kinase and BLNK adaptor, resulting in suppression of pre–BCR signaling. This regulatory circuit prevents the pre–BCR from inducing additional Igl chain gene rearrangements and driving pre–B cells with RAG DSBs into cycle. We propose that pre–B cells toggle between pre–BCR signals and a RAG DSB–dependent checkpoint to maintain genome stability while iteratively assembling Igl chain genes.

Developing B cells must assemble and express genes encoding the immunoglobulin heavy (Igh) and light (Igl) chains of the B cell receptor (BCR; Rajewsky, 1996). This process occurs through V(D)J recombination, a reaction that assembles the second exon of lymphocyte antigen receptor genes from component variable (V), joining (J), and, at some loci, diversity (D) gene segments (Fugmann et al., 2000). V(D)J recombination is initiated when the RAG endonuclease, composed of RAG-1 and RAG-2, introduces DNA double-strand breaks (DSBs) at a pair of recombining gene segments, generating two blunt signal DNA ends and two hairpin–sealed coding DNA ends (Fugmann et al., 2000). RAG DSBs are generated in G1 phase lymphocytes, where they activate the ataxia–telangiectasia mutated (ATM) DNA damage response (DDR) kinase (Desiderio et al., 1996; Helmink and Sleckman, 2012). In G1-phase cells, ATM inhibits S–phase entry by initiating canonical cell cycle checkpoint pathways through induction of p53 and directs DSB repair by nonhomologous end joining (NHEJ; Shiloh, 2003; Helmink and Sleckman, 2012). In addition, in response to RAG DSBs, ATM activates a genetic program that may function to regulate processes required for normal lymphocyte development (Bredemeyer et al., 2008; Bednarski et al., 2012; Helmink and Sleckman, 2012; Steinel et al., 2013).

B cell development requires the sequential assembly of Igh chain genes in pro–B cells and Igl chain genes (Iglκ [Igk] or Iglλ [IgL]) in pre–B cells (Rajewsky, 1996). The ordered assembly of immunoglobulin receptor genes is directed by signals from cell surface receptors. The IL–7r signals through AKT and JAK–STAT pathways to promote survival and to regulate Igh chain gene rearrangement in pro–B cells (Bertolino et al., 2005; Clark et al., 2014). Productive assembly of an Igh chain gene leads to its expression with the surrogate light chain (λ5 and Vpre–B) and the CD79A–CD79B heterodimer (Igα and Igβ, respectively) to generate the pre–BCR (Herzog et al., 2009; Rickert, 2013). Oligomerization of the pre–BCR, through ligand–dependent or –independent mechanisms, activates the SYK tyrosine kinase, leading to phosphorylation of the adaptor protein BLNK (also known as SLP–65; Herzog et al., 2009; Rickert, 2013).

Pre–BCR signals, along with those from the IL–7r, promote the developmental transition of pro–B cells to rapidly cycling, large pro–B cells (Herzog et al., 2009; Rickert, 2013; Clark et al., 2014). Pre–BCR and IL–7r signals synergize to

*J.J. Bednarski and R. Pandey contributed equally to this paper.
Correspondence to Barry P. Sleckman: bas2022@med.cornell.edu

Abbreviations used: ATM, ataxia–telangiectasia mutated; BCR, B cell receptor; ChIP, chromatin immunoprecipitation; DDR, DNA damage response; DSB, double-strand break; NHEJ, nonhomologous end joining.

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drive proliferation, whereas they independently regulate differentiation and survival, respectively. Activation of STAT5 by the IL-7r signaling leads to increased SYK and BLNK expression, which can affect the localization of pre-B cells with respect to IL-7–producing stromal cells (Tokoyoda et al., 1994; Johnson et al., 2008). Moreover, activation of RAS by the pre-BCR in large pre-B cells promotes exit from the cell cycle (Mandal et al., 2009). Loss of IL-7r signaling leads to increased SYK and BLNK expression, which reinforces pre-BCR signaling (Ochiai et al., 2012).

Pre-BCR signals are required to initiate Igk chain gene assembly through activation of transcription factors and histone modifications that regulate Igk accessibility and RAG recruitment (Clark et al., 2014). The pre-BCR induces expression of IRF4, which, together with PU.1, binds the 3’ Igk enhancer to promote Igk germline transcription and rearrangement (Pongubala et al., 1992; Johnson et al., 2008; Clark et al., 2014). Small pre-B cells often undergo multiple sequential Igk rearrangements over several days as they attempt to generate a functional Igk chain gene (Casellas et al., 2001). Once RAG DSBs are generated, the pre-BCR must be prevented from initiating additional Igk rearrangements. Moreover, activation of SYK by the pre-BCR could drive small pre-B cells with RAG DSBs into cycle (Rolink et al., 2000; Wossing et al., 2006; Herzog et al., 2009; Rickert, 2013). In pre-B cells, RAG DSBs activate canonical cell cycle checkpoint pathways, including p53 (Guidos et al., 1996; Helmink and Sleckman, 2012). However, in other cell types checkpoint pathways, including p53 (Guidos et al., 1996; Rickert et al., 2000; Wossing et al., 2006; Herzog et al., 2009; Rickert et al., 2014). IL-7r signaling leads to increased SYK and BLNK expression, which reinforces pre-BCR signaling (Ochiai et al., 2012).

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We reasoned that pre-BCR signaling must be regulated to order Igk chain gene assembly and prevent these signals from driving pre-B cells with RAG DSBs into cycle. Indeed, we show here that RAG DSBs activate a cell type-specific checkpoint pathway that inhibits pre-BCR signaling. This checkpoint pathway suppresses SYK and BLNK expression, inactivating pre-BCR signals to both prevent cell cycle progression and regulate Igk chain gene assembly. We propose that pre-B cells toggle between pre-BCR signaling and this RAG DSB-dependent checkpoint pathway, allowing for iterative Igk chain gene assembly while maintaining genome stability.

RESULTS
RAG DSB signals regulate the genetic program of small pre-B cells
To elucidate the pre-B cell genetic program regulated by RAG DSB signals, we used mice deficient in RAG-1 or the Artemis endonuclease that express the μIgh and Bcl2 transgenes (Rag1−/−μIgh:Bcl2 and Art−/−μIgh:Bcl2, respectively; Bednarski et al., 2012). The μIgh transgene enables formation of a pre-BCR, allowing for pre-B cell development in these mice, and the Bcl2 transgene supports pre-B cell survival in vitro (Bednarski et al., 2012). Culturing bone marrow from these mice in the presence of IL-7 leads to the selective expansion of large pre-B cells (Rolink et al., 1991; Johnson et al., 2008; Bednarski et al., 2012). Upon withdrawal of IL-7, these cells transit to the small pre-B cell stage and induce RAG expression (Bednarski et al., 2012; Steinel et al., 2013; Fig. S1). Rag1−/−μIgh:Bcl2 small pre-B cells do not generate RAG DSBs. Art−/−μIgh:Bcl2 small pre-B cells generate RAG DSBs at Igk, but these DSBs are not repaired because Artemis is required to open hairpin-sealed coding DNA ends (Helmink and Sleckman, 2012; Fig. 1 A). These DSBs activate DDR, as indicated by phosphorylation of p53 (Fig. 1 B).

Gene profiling revealed that ∼3,000 genes change expression (>1.5-fold) in Art−/−μIgh:Bcl2 or Rag1−/−μIgh:Bcl2 pre-B cells as they transition from large (in IL-7) to small (IL-7 withdrawal) pre-B cells (Fig. 1, C–E; and Table S1). Many of these gene expression changes are observed during B cell development in vivo (Heng and Painter, 2008; Fig. 1 F). Comparison of Art−/−μIgh:Bcl2 and Rag1−/−μIgh:Bcl2 small pre-B cells reveals that RAG DSB signals regulate a significant fraction (∼40%) of the gene expression changes that occur during the transition of large to small pre-B cells (RAG DSB dependent; Fig. 1, C–E; and Table S1).

ATM and NIK activate NF-κB in small pre-B cells
In small pre-B cells, RAG DSBs induce expression of the Relb and NIKb2 (p100) genes, which encode the principal components of the noncanonical NF-κB (NF-κB2) pathway (Hayden and Ghosh, 2012; Sun, 2012; Fig. 2 A and Table S1). This depends on the activation of ATM as revealed by analyses of Art−/−Atm−/−μIgh:Bcl2 small pre-B cells in vitro and Atm−/−pre-B cells in vivo (Fig. 2, A and B). The p100 and RELB proteins are induced by RAG DSBs (compare Art−/−μIgh:Bcl2 and Rag1−/−μIgh:Bcl2) in small pre-B cells that express ATM and have intact DDR signals (compare Art−/−μIgh:Bcl2 and Art−/−Atm−/−μIgh:Bcl2; Fig. 2 C). Formation of the transcriptionally active p52-RELB heterodimer, which translocates to the nucleus, requires cleavage of p100 to form p52 (Hayden and Ghosh, 2012; Sun, 2012). In this regard, the robust generation of p52 and the nuclear translocation of p52 and RELB are observed only in small pre-B cells with RAG DSBs (Fig. 2, C and D). In mature B cells, cleavage of p100 to form p52 depends on the NF-κB-inducing kinase (NIK; Hayden and Ghosh, 2012; Sun, 2012). Similarly, in small pre-B cells NIK is required.
for the generation of p52, as Art−/−:Nik−/−:μIgh:Bcl2 small pre-B cells show induction of p100 in response to RAG DSBs, but inefficient conversion of p100 to p52 (Fig. 2 E).

Thus, NF-κB2 is activated in small pre-B cells through the RAG DSB-dependent induction of p100 and RELB coupled with the NIK-dependent conversion of p100 to p52.
NF-κB2 induces SPIC expression

Analysis of Art^{-/-};μIgh:Bcl2 and Art^{-/-};Nfkbo2^{-/-};μIgh:Bcl2 small pre–B cells revealed that ~15% of the genes regulated by RAG DSBs depend on NF-κB2 (Fig. 3 A and Table S2). One of these genes encodes SPIC, an ETS family transcription factor with significant homology to PU.1 and SPIB (Bemark et al., 1999; Hashimoto et al., 1999). Induction of Spic in response to RAG DSBs depends on ATM and NF-κB2, as indicated by analysis of Art^{-/-};μIgh:Bcl2, Art^{-/-};Atm^{-/-};μIgh:Bcl2, and Art^{-/-};Nfkbo2^{-/-};μIgh:Bcl2 small pre–B cells (Fig. 3 B). To assess SPIC expression in vivo, we used mice with a Spic reporter allele, Spic^{igfp}, which contains an IRES-EGFP targeted to a 3' noncoding exon of the Spic gene (Haldar et al., 2014). Approximately 40% of bone marrow small pre–B cells from Art^{-/-};Spic^{igfp};μIgh:Bcl2 mice express EGFP (Fig. 3 C). CD40 surface expression is induced by RAG DSB signals, and thus functions as a marker of small pre–B cells with RAG DSBs (Bredemeyer et al., 2008). All of the EGFP-positive small pre–B cells in Art^{-/-};Spic^{igfp};μIgh:Bcl2 mice also express CD40 (Fig. 3 C). In contrast, none of the small pre–B
cells from Rag1<sup>−/−</sup>:Spic<sup>ΔEts/ΔEts</sup>μIgh:Bcl2 mice express EGFP or CD40 (Fig. 3 C). Together, these data are consistent with the interpretation that SPIC expression in small pre–B cells is induced by RAG DSBs through the sequential activation of ATM and NF-κB2.

RAG DSBs negatively regulate pre–BCR signaling

Ectopic SPIC expression in mature B cells inhibits BCR signaling, and in lymphocyte progenitors it blocks B cell development at the pre–B cell stage (Zhu et al., 2008). As RAG DSBs induce SPIC, we reasoned that they might negatively regulate pre–BCR signaling. Indeed, we find that expression of both SYK and BLNK is reduced in small pre–B cells with RAG DSBs (Art<sup>−/−</sup>μIgh:Bcl2) but not in those without RAG DSBs (Rag1<sup>−/−</sup>:μIgh:Bcl2) or in ATM-deficient (Art<sup>−/−</sup>:Atm<sup>−/−</sup>μIgh:Bcl2) pre–B cells with RAG DSBs (Fig. 4, A–D). Consistent with the reduction in these proteins, small pre–B cells with RAG DSBs have decreased pre–BCR signaling as evidence by reduced levels of phosphorylated BLNK (Fig. 4 A). Expression of BTK, another pre–BCR signaling protein, is not affected by RAG DSBs but is minimally decreased by loss of IL–7r signals (Fig. 4, E and F). Retroviral expression of SPIC in Rag1<sup>−/−</sup>:μIgh:Bcl2 small pre–B cells leads to inhibition of both Blnk and Syk expression (Fig. 5 A). Moreover, SPIC-expressing (EGFP-positive) small pre–B cells in Spic<sup>ΔEts/ΔEts</sup> mice have lower levels of Syk and Blnk transcripts as compared with cells that do not express SPIC (EGFP negative; Fig. 5 B). We conclude that RAG DSBs antagonize the expression of two key pre–BCR signaling proteins, SYK and BLNK, through the sequential induction of ATM, NF-κB2, and SPIC.

SPIC antagonizes PU.1 binding at Syk and Blnk

The expression of Blnk depends on PU.1 binding to its promoter (Schweitzer and DeKoter, 2004; Xu et al., 2012). SPIC functions as a transcriptional repressor that recognizes the same DNA sequence as PU.1 but, unlike PU.1, SPIC is unable to bind IRF4, which is required to initiate transcription (Bemark et al., 1999; Hashimoto et al., 1999; Carlsson et al., 2003). To determine whether SPIC induction by RAG DSBs affects PU.1 activity, we compared PU.1 binding at Blnk in Art<sup>−/−</sup>μIgh:Bcl2 and Rag1<sup>−/−</sup>μIgh:Bcl2 small pre–B cells. Although PU.1 levels are similar in these cells, binding of PU.1 to the Blnk promoter is markedly reduced in Art<sup>−/−</sup>μIgh:Bcl2 small pre–B cells, which have RAG DSBs and up-regulate SPIC, as compared with Rag1<sup>−/−</sup>μIgh:Bcl2 small pre–B cells that do not express SPIC (Fig. 6, A and B). When SPIC is retrovirally expressed in Rag1<sup>−/−</sup>μIgh:Bcl2 small pre–B cells, we observe SPIC binding to both the Blnk promoter and a putative regulatory element upstream of the Syk gene (Zhang et al., 2012; Fig. 6, C and D). Moreover, we also observed decreased PU.1 binding at these regulatory elements in Rag1<sup>−/−</sup>μIgh:Bcl2 small pre–B cells expressing SPIC (Fig. 6 E). Mutation of the conserved Ets domain (R175G) in SPIC (SPIC<sup>R175G</sup>) ablates SPIC binding to Blnk and Syk, similar to previous findings for PU.1 (Pio et al., 1996; Suraweera et al., 2005; and unpublished data). Compared with SPIC, equivalent expression of SPIC<sup>R175G</sup> in Rag1<sup>−/−</sup>μIgh:Bcl2 small pre–B cells does not result in decreased PU.1 bind-
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SPIC inhibits germline transcription and RAG cleavage at Igk

SYK and BLNK signals from the pre–BCR induce IRF4 to promote germline transcription and rearrangement of the Igk locus. Inhibition of pre–BCR signaling by RAG DSBs could feed back to suppress this process. Indeed, small pre–B cells with RAG DSBs (Art−/−:μIgh:Bcl2) exhibit decreased IRF4 expression and Igk germline transcripts as compared with Rag1−/−:μIgh:Bcl2 small pre–B cells (Fig. 7, A and B). Moreover, retroviral expression of SPIC in Rag1−/−:μIgh:Bcl2 small pre–B cells leads to a reduction in both Irf4 and germline Igk transcripts (Fig. 7 C). Germline Igk transcription requires PU.1 binding at 3′ Igk enhancer, which is significantly reduced in Art−/−:μIgh:Bcl2 small pre–B cells with RAG DSBs as compared with Rag1−/−:μIgh:Bcl2 small pre–B cells (Fig. 7 D). Moreover, when SPIC is retroviral expressed in Rag1−/−:μIgh:Bcl2 small pre–B cells,

Figure 4. RAG DSBs suppress pre–BCR signaling. (A) Western blot of phosphorylated BLNK (p-BLNK) and total BLNK in pre–B cells in IL-7 (0 d) and following IL-7 withdrawal (IL-7 wd) for the indicated days. GAPDH is shown as a protein loading control. (B and C) Western blot of SYK in pre–B cells cultured in IL-7 (+) and 3 d after IL-7 withdrawal (-). GAPDH is shown as a protein loading control. (D and E) Blnk, Syk and Btk mRNA expression in Rag1−/−:μIgh:Bcl2 (black), Art−/−:μIgh:Bcl2 (red), and Art−/−:Atm−/−:μIgh:Bcl2 (blue) small pre–B cells 3 d after IL-7 withdrawal. Data are mean and standard error for three replicates. (F) Western blot of BTK in pre–B cells cultured in IL-7 (+) and 3 d after IL-7 withdrawal (-). GAPDH is shown as a protein loading control. Data in A, B, C, and F are representative of at least three independent experiments. *, P ≤ 0.05; ***, P ≤ 0.005; ****, P ≤ 0.0001 by Student’s t test.
we observe SPIC binding at the 3′ Igk enhancer and a concomitant decrease in PU.1 binding (Fig. 7, E and F). Thus, SPIC negatively regulates germline Igk transcription both by inhibiting pre–BCR signals that lead to IRF4 expression and by interfering with PU.1 binding at the 3′ Igk enhancer.

The activation of ATM by Igk rearrangements feeds back to inhibit the generation of additional RAG DSBs as indicated by increased RAG cleavage at Igk in Art−/−:Atm−/−:μIgh:Bcl2 as compared with Art−/−:μIgh:Bcl2 small pre–B cells (Hewitt et al., 2009; Steinel et al., 2013; Fig. 1 A). Retroviral expression of SPIC in Art−/−:μIgh:Bcl2 small pre–B cells results in decreased RAG cleavage at Igk (Fig. 7 G). This is indicated by the increase in Igk loci in the germline configuration and a concomitant decrease in Igk coding ends in Art−/−:Atm−/−:μIgh:Bcl2 small pre–B cells expressing SPIC as compared with Art−/−:Atm−/−:μIgh:Bcl2 small pre–B cells expressing the empty retroviral vector (Fig. 7 G). We conclude that the ATM-dependent induction of SPIC by RAG DSBs in small pre–B cells feeds back to inhibit germline transcription and the generation of additional RAG DSBs at the Igk locus.

DISCUSSION

Here, we show that RAG DSBs activate a cell type–specific checkpoint pathway that functions to both enforce G1 cell cycle arrest and regulate Igk chain gene rearrangement in small pre–B cell. This occurs through the sequential activation of ATM and NF-κB2, which leads to induction of SPIC, a hematopoietic-specific transcriptional repressor. SPIC inhibits pre–BCR signaling by repressing the expression of SYK and BLNK. Additionally, SPIC directly antagonizes Igk locus germline transcription and rearrangement. Thus, signals from RAG DSBs generated during Igk gene assembly are integrated into the signaling circuitry of developing pre–B cells.

In mature B cells, p100 and RELB are constitutively expressed and NF-κB2 activation occurs upon engagement of CD40 or BAFF receptors with their respective ligands, leading to stabilization of NIK and conversion of p100 to p52. In resting cells, NIK exists in a complex with cIAP ubiquitin ligases, which mediate NIK degradation (Vallabhapurapu et al., 2008; Zarnegar et al., 2008; Hayden and Ghosh, 2012; Sun, 2012). Signals from the CD40 or BAFF receptors
trigger dissociation of this complex and increased NIK levels (Vallabhapurapu et al., 2008; Zarnegar et al., 2008; Hayden and Ghosh, 2012; Sun, 2012). Similar to mature B cells, pre–B cells require NIK to promote the conversion of p100 to p52 in response to RAG DSBs. However, in contrast to mature B cells, expression of p100 and RELB must be induced in pre–B cells, and this depends on the activation of ATM by RAG DSBs generated during Igk chain gene assembly. Thus, pre–B cells require the convergence of two signals (ATM and NIK) to activate NF-κB2.

In response to RAG DSBs, NF-κB2 induces the expression of SPIC, an ETS family transcription factor with homology to PU.1 and SPIB (Bemark et al., 1999; Hashimoto et al., 1999). PU.1 and SPIB are constitutively expressed in developing B cells, where they perform critical developmental functions (Schweitzer and DeKoter, 2004). In mature B cells, SPIC regulates BCR signaling and canonical NF-κB1 activation (Zhu et al., 2008; Li et al., 2015). How SPIC is regulated and functions in early B cells has not been determined. Here, we show that in developing B cells, SPIC is induced by RAG DSBs. Consequently, SPIC is only expressed in small pre–B cells that are actively undergoing Igk chain gene rearrangement. Thus, pre–B cells require the convergence of two signals (ATM and NIK) to activate NF-κB2.

In large pre–B cells, signals from the pre–BCR attenuate IL-7r signals, which is required for transition to the small pre–B cell stage and for initiation of Igk chain gene assembly (Rolink et al., 1991; Johnson et al., 2008; Mandal et al., 2009; Ochiai et al., 2012; Clark et al., 2014). The pre–BCR
signals through BLNK to antagonize AKT signaling down-stream of the IL-7r (Herzog et al., 2008; Ochiai et al., 2012). Attenuation of IL-7r signaling leads to increased expression of SYK and BLNK, which further augments pre–BCR signaling (Ochiai et al., 2012). We show that once Igk chain gene assembly has been initiated, RAG DSB signals feedback to inhibit the pre–BCR by suppressing the expression of SYK and BLNK, which could lead to reactivation of IL-7r signals. However, pre–BCR signals may also function to re-position small pre–B cells to bone marrow niches devoid of
IL-7-producing stromal cells (Tokoyoda et al., 2004; John-son et al., 2008). Moreover, in small pre–B cells, RAG DSBs induce CD69, SWAP-70, and l-selectin, which function in lymphocyte trafficking and localization (Bredemeyer et al., 2008). This could enforce localization of pre–B cells in IL-7 poor areas of the bone marrow once Igk chain assembly has initiated. RAG DSBs also induce the PIM2 kinase, which antagonizes IL-7r signals (Bednarski et al., 2012). Thus, although pre–BCR signaling is required for the initial suppression of IL-7r signaling in small pre–B cells,
continued pre–BCR signaling may not be required to maintain this suppression.

Initiation of pre–BCR signaling occurs upon the activation of SYK, which phosphorylates BLNK (Herzog et al., 2009; Rickert, 2013; Clark et al., 2014). We find that RAG DSBs inactivate pre–BCR signaling by inhibiting both SYK and BLNK. Why would it be important for RAG DSBs to inhibit both proteins? In the absence of SYK, ZAP–70 can phosphorylate BLNK, activating downstream signaling cascades (Chen et al., 2005; Fallah-Arani et al., 2008). Thus, the isolated inhibition of SYK would not attenuate pre–BCR signaling, BLNK antagonizes SYK activation by the pre–BCR (Flemming et al., 2003; Taguchi et al., 2004). Consequently, the isolated loss of BLNK increases SYK activation (Flemming et al., 2003; Taguchi et al., 2004). Indeed, both loss of BLNK and overactivation of SYK are associated with B cell transformation and leukemia (Flemming et al., 2003; Taguchi et al., 2004; Perova et al., 2014; Geng et al., 2015). Thus, complete suppression of pre–BCR signaling by RAG DSBs would rely on the inactivation of both SYK and BLNK. Here, we have defined the signaling pathway that inhibits pre–BCR signaling under physiological conditions in normal pre–B cells. Conceivably, this pathway could be therapeutically targeted in pre–B cell leukemias that depend on pre–BCR signaling.

DNA DSBs activate p53-dependent canonical checkpoint pathways to prevent cell cycle progression until the DSBs have been repaired (Shiloh, 2003). However, in some cell types proliferative signals can bypass p53-mediated checkpoints, driving cells with DSBs through cell cycle and promoting genome instability (Quelle et al., 1998; Sitko et al., 2008). RAG DSBs activate p53 in G1–phase small pre–B cells (Gonzales et al., 1996). However, we find that even in the absence of p53, small pre–B cells with RAG DSBs are still arrested in G1. Furthermore, restoration of pre–BCR signaling through SYK expression overrides the p53-mediated checkpoint leading to the S-phase progression of small pre–B cells with RAG DSBs. Thus, enforcement of the G1–S checkpoint in small pre–B cells depends on activation of both the canonical checkpoint (p53) and a cell type–specific checkpoint pathway that inhibits pre–BCR proliferative signals. In the absence of ATM, these checkpoint pathways are defective. Consequently, ATM-deficient pre–B cells with RAG DSBs enter cell cycle and persist in the periphery for several weeks (Callén et al., 2007).

Small pre–B cells undergo multiple rounds of Igδ chain gene rearrangement over a span of several days as they attempt to generate a productive Igδ chain that can pair with the Igα chain to form a nonautoreactive BCR (Rajewsky, 1996; Casellas et al., 2001). Pre–BCR signals initiate Igδ chain rearrangement through induction of IRF4 and subsequent activation of germline Igk transcription (Johnson et al., 2008; Clark et al., 2014). Once RAG DSBs are generated in the Igk locus, additional RAG cleavage events must be inhibited. In this regard, we show that RAG DSBs suppress pre–BCR signaling, leading to a reduction in Igk germline transcrip-

tion and inhibition of additional DSBs at the Igk locus. This occurs through the ATM– and NF-kB2–dependent induction of SPIC, which inhibits SYK and BLNK and interferes with PU.1 binding to the Igk enhancer. This feedback circuit prevents pre–B cells with RAG DSBs from entering cycle and prevents additional RAG DSBs at the Igk locus until the initial rearrangement has been completed and ATM signaling is terminated. If this rearrangement encodes an Igk chain that forms a functional BCR, the cell transits to the immature B cell stage. However, if the Igk chain gene is not functional, loss of ATM signaling would lead to the reexpression of SYK and BLNK, leading to reactivation of pre–BCR signaling and induction of another Igk chain gene rearrangement. We propose that small pre–B cells toggle between pre–BCR signals and a RAG DSB-dependent cell-type specific checkpoint to order iterative Igδ chain gene rearrangements and maintain genomic stability by preventing pre–B cells with RAG DSBs from entering cell cycle.

**MATERIALS AND METHODS**

**Mice.** All mice were bred and maintained under specific pathogen–free conditions at the Washington University School of Medicine and were handled in accordance to the guidelines set forth by the Division of Comparative Medicine of Washington University. Wild-type B6 mice (The Jackson Laboratory) were used as controls. p53−/− mice were purchased from The Jackson Laboratory. Spic−/−/−, Nik−/−, and Nikk2−/− mice were generated as previously described (Yin et al., 2001; Zarnegar et al., 2004; Haldar et al., 2014). Spic−/−/− mice were on a B6 background. All other mice are on a mixed genetic background. In vivo studies were conducted on 4–5-wk-old mice.

**Cell culture.** Primary pre–B cell cultures were generated from bone marrow harvested from 4–6-wk-old mice and cultured for 7–10 d at 2 × 10⁶ cells/ml in media containing 5 ng/ml of IL–7 (Life Technologies). For IL–7 withdrawal experiments, cells were resuspended in media without IL–7 and maintained at 2 × 10⁶ cells/ml.

**Retroviral transduction and cDNA expression.** pMSCV-mCherry-SYK was purchased from Addgene (plasmid 50045; Strijbis et al., 2013). cDNA encoding SPIC with a 5′ Flag tag was cloned into the pOZ retrovirus containing an IRES sequence, followed by truncated human CD25 (hCD25) cDNA as a marker of transduced cells. A DNA binding-deficient SPIC was generated by mutating arginine 175 to glycine (R175G) using QuikChange II XL (Agilent) according to the manufacturer’s protocol (Pio et al., 1996; Suraweera et al., 2005). Retrovirus was produced in platE cells by transfection of the retroviral plasmid with Lipofectamine 2000 (Life Technologies) according to the manufacturer’s protocol. Viral supernatant was collected and pooled from 24–72 h after transfection. Viral supernatant was used immediately to transduce cells or was concentrated before transduction. To concentrated viral...
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particles, PEG-8000 (Sigma-Aldrich; final concentration 8%) was added to viral supernatant, incubation at 4°C overnight, and centrifugation at 2,500 RPM for 20 min. Precipitated virus was resuspended at 300× concentration in sterile PBS. Pre–B cells were transduced with unconcentrated virus (40 × 10⁶ cells in 3 ml viral supernatant) or with concentrated virus (40 × 10⁶ in 1 ml with 1× viral particles) in IL-7–containing media with polybrene (5 µg/ml; Sigma-Aldrich) by centrifugation for 2 h at 1,200 RPM at room temperature. 4 h later, fresh IL-7 media (2 ml) was added, and the cells were incubated overnight. Virus-containing media was removed and cells were cultured in fresh IL-7 media (2 × 10⁶/ml). Cells expressing the retrovirus construct were identified by flow cytometric assessment of hCD25 or mCherry expression using a FACSCalibur (BD). Transduced cells were sorted using biotin-conjugated anti–hCD25 (BD) and anti-biotin magnetic beads (Miltenyi Biotec) on MS columns (Miltenyi Biotec) according to the manufacturer’s protocol.

Flow cytometric analyses and cell sorting. Flow cytometric analyses were performed on a FACSCalibur or BD LSRII Fortessa (BD). Sorting was conducted on a BD Aria (user-operated) or on a Sony Sy3200 through the Siteman Cancer Center Flow Cytometry Core Facility. FITC-conjugated anti-CD45RA/B220 (clone RA3-6B2), phycoerythrin (PE)-conjugated anti-CD43 (clone S7), FITC-conjugated anti-CD43 (clone S7), PE-Cy7–conjugated anti-CD45/B220 (clone RA3-6B2), allophycocyanin (APC)-conjugated anti-IgM (clone II/41), PE-conjugated anti-CD40 (clone 3D10), PE-conjugated anti-CD43 (clone S7), PE-Cy7–conjugated anti-CD45/R/B220 (clone RA3-6B2), phycoerythrin (PE)-conjugated anti-CD43 (clone S7), and Streptavidin was purchased from BD. APC-conjugated streptavidin was purchased from eBioscience. PE-conjugated anti-hCD25 (clone BC96) and APC-conjugated anti-hCD25 (clone BC96) were purchased from BioLegend. APC-conjugated anti-mCherry (clone 16D7) was purchased from Life Technologies. Staining for intracellular proteins was performed using Cytofix/Cytoperm solution (BD).

Cell cycle analysis. To assess pre–BCR driven proliferation, pre–B cells were resuspended in media without IL-7 and maintained at 2 × 10⁶ cells/ml. 8 h after removal from IL-7, cells were pulsed BrdU for 2 h using the BrdU-FITC kit (BD) per the manufacturer’s instructions. DNA content was assessed by 7AAD (BD) or DAPI (Sigma-Aldrich). The SYK (BD) per the manufacturer’s instructions. DNA content was assessed by 7AAD (BD) or DAPI (Sigma-Aldrich). The SYK

Cell fractionation and Western blot. Cell fractionation was performed as previously described (Méndez and Stillman, 2000). Western blots were done on whole-cell lysates as previously described (Bredemeyer et al., 2008). Anti–NF-kB2 (recognizes p100 and p52; polyclonal), anti–RELB (clone C1E4), anti–BLNK (clone D8R3G), anti–SYK (clone D115Q), anti–BTK (clone D3H5), and anti–phospho(S15)-p53 (polyclonal) antibodies were purchased from Cell Signaling Technology. Anti–TRA2F (polyclonal), anti–TRA3 (polyclonal), anti–IRF4 (clone E-7), anti–PU.1 (polyclonal), and anti–LaminB (clone G-1) were obtained from Santa Cruz Biotechnology. Anti-phosphorylated BLNK (clone J117-1278) was purchased from BD. Anti–GAPDH (clone GAP DH-71.1) and anti–FLAG (clone M2) were from Sigma-Aldrich. Secondary reagents were horseradish peroxidase (HRP)–conjugated anti–mouse IgG (Cell Signaling Technology) or anti–rabbit IgG (Cell Signaling Technology). Western blots were developed with ECL (Thermo Fisher Scientific) and ECL Prime (GE Healthcare).

Southern blot. Southern blot analyses of coding ends generated during rearrangement at the Igk locus were performed on genomic DNA digested with SacI and EcoRI using the JkIII probe and Tcrb probe, as described previously (Bredemeyer et al., 2008).

RT-PCR. RNA was isolated using RNeasy (Qiagen) and reversed transcribed using either a polyT primer or a random hexamer primer with SuperScriptII (Life Technologies) according to the manufacturers’ protocol. RT-PCR was performed using Brilliant II SYBR Green (Agilent) according to the manufacturer’s protocols. Data have been deposited in NCBI’s Gene Expression Omnibus under accession no. GSE67854. Differentially expressed genes were identified by error-weighted analysis of variance (ANOVA; Partek Genomics Suite v6.12). Fold changes were calculated based on the mean of three cell lines for each genotype. Only those genes with a fold change of ≥1.5 and a p-value ≤ 0.05 were considered for further analysis. ImmGen (Immunological Genome project) project gene expression data were independently analyzed to identify all genes expression changes that were significantly changed (P < 0.05) between large and small pre–B cells (datasets: preB_FrC_BM vs. preB_FrD_BM; Heng and Painter, 2008).

Chromatin immunoprecipitation (ChIP). ChIP was performed using anti–PU.1 (Santa Cruz Biotechnology), anti–FLAG (Sigma–Aldrich), control rabbit IgG (Santa Cruz Biotechnology), and control mouse IgG antibodies (eBioscience) as previously described (Ochiai et al., 2013). In brief, DNA from 5 × 10⁶ cells was cross-linked with 1% formaldehyde for 10 min at room temperature Reaction was stopped with 125 µM Glycine. Cells were lysed with NP-40 and nuclei were frozen.
in liquid nitrogen, and then lysed with SDS. DNA was sonicated with 30-s pulses for 60–90 cycles. DNA fragmentation was in the range of 0.5–1 kb and was monitored by agarose gel electrophoresis. Immunoprecipitation was performed with 1 µg of anti-PU.1 (polyclonal; Santa Cruz Biotechnology) or control rabbit IgG (clone P3.6.2.8.1; EMD Millipore) and immunopurification was performed with Protein A Dynabeads (Life Technologies). DNA was purified with QIAquick PCR purification kit (QIAGEN). Quantitative PCRs was performed using Brilliant II SYBR Green (Agilent) and acquired on an Mx3000P (Stratagene). Primers are listed in Table S3.

Statistical analysis. For analyses other than gene array, p-values were generated via Student’s t test (unpaired, two-tailed) using Prism (GraphPad Software).

Online supplemental material. Fig. S1 shows IL-7 culture system for studying RAG DSBs. Table S1 shows genes regulated by RAG DSBs. Table S2 shows genes regulated by NF-kB2. Table S3 shows sequences of primers. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20151048/DC1.

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